ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochemical and Biophysical Research Communications 301 (2003) 338–343

BBRC

www.elsevier.com/locate/ybbrc

Chondrogenesis enhanced by overexpression of *sox9* gene in mouse bone marrow-derived mesenchymal stem cells

Hiroki Tsuchiya,* Hiroshi Kitoh, Fumiaki Sugiura, and Naoki Ishiguro

Department of Orthopaedic Surgery, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

Received 10 December 2002

Abstract

We investigated chondrogenesis of cell-mediated *sox9* gene therapy as a new treatment regimen for cartilage regeneration. pIRES2-EGFP vector containing a full-length mouse *sox9* cDNA was transfected into bone marrow-derived mesenchymal stem cells (MSCs) by lipofection and chondrogenic differentiation of these cells was evaluated. In vitro high density micromass culture of these *sox9* transfected MSCs demonstrated that a matrix-rich micromass aggregate with EGFP expressing MSCs was positively stained by Alcian blue and type II collagen. Next, *sox9* transfected MSCs were loaded into the diffusion chamber and transplanted into athymic mice to analyze in vivo chondrogenesis. A massive tissue formation in about 2 mm diameter was visible in the chamber after 4 weeks transplantation. Histological examinations demonstrated that both Alcian blue and type II collagen were positively stained in the extracellular matrix of the mass while type X collagen was not stained. These results indicated that cell-mediated *sox9* gene therapy could be a novel strategy for hyaline cartilage damage.

Keywords: *Sox9*; Mesenchymal stem cell; Lipofection; Chondrogenic differentiation

Articular cartilage is an avascular tissue composed of hyaline cartilage with both functions as a shock absorber and provides a smooth joint movement. It is a complex tissue containing a unique set of extracellular matrix molecules including type II, IX, and XI collagens and aggrecan. The composition of the cartilage extracellular matrix is governed by chondrocytes which express cartilage-specific genes. Articular cartilage damaged by trauma or degenerative arthritis has a limited capacity for regeneration most likely because of its avascularity and low cellularity. Treatment of full-thickness defect of the cartilage is still a difficult and troublesome problem for orthopedic surgeons [1].

Cell based therapy, that is, implantation of cells or engineered cartilage, may represent an alternative approach to articular cartilage damage. Brittberg et al. [2] reported that the cartilaginous defect in the knee was successfully treated with transplantation of autologous chondrocytes cultured under a monolayer culture condition to increase the number of chondrocytes. Previous studies, however, demonstrated that chondrocytes in monolayer culture do not maintain the chondrocyte phenotype morphologically or biochemically [3,4]. In prolonged monolayer culture, the morphology of chondrocytes changes to that of fibroblast-like cells, suggesting dedifferentiation.

Autologous chondrocyte transplantation has a limited clinical application for a relatively large cartilage defect because it is difficult to obtain sufficient amount of cells for an occurrence of the new cartilage defect at the donor site and the nature of hypocellularity in the cartilage tissue. Tissue engineering, that is, small biopsy specimens from the relatively uninvolved sites can be obtained from the patient, and cells can be isolated, grown, and expanded in culture in large numbers, may overcome these problems. The in vitro expansion of donor material significantly reduces the amount of biopsy material required and the morbidity of the donor site. Bone marrow-derived mesenchymal stem cells (MSCs) have the potential to form a variety of mesenchymal tissues including bone, cartilage, tendon,

* Corresponding author. Fax: +81-52-744-2260.
E-mail address: hiroki-t@gc4.so-net.ne.jp (H. Tsuchiya).

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(02)03026-7

ligament, muscle, and fat [5–10]. MSCs are appropriate for donor tissue in tissue engineering and may be used in clinical applications because they are easily isolated from a small aspirate of bone marrow and readily generate various cell types.

In vitro studies of chondrogenesis can be designed to distinguish the effects of specific signals from the complex factors present in vivo. *Sox9* is a member of the family of Sox (Sry-type HMG box) genes and has been shown to be expressed predominantly in mesenchymal condensations and cartilage [11]. *Sox9* regulates expression of the gene encoding type II procollagen, the major matrix protein characteristic of chondrocytes [12,13]. In addition, *sox9* enhances aggrecan gene promotor/enhancer activity in a cartilage-derived cell line [14]. These observations indicate that *sox9* plays a key role in chondrogenesis and skeletogenesis [15].

In this study, full-length *sox9* cDNA was transfected into culture-expanded mouse MSCs. Chondrogenesis both in vitro and in vivo induced by overexpressed *sox9* gene suggested that cell-mediated *sox9* gene therapy could be a novel treatment for orthopaedic diseases associated with hyaline cartilage damage.

Materials and methods

Cell isolation and culture. The femora of the young male mice of Std:ddy, aged 28–30 days and weighing 25–30 g, were excised aseptically, cleaned of soft tissues, and passed through with Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10× the usual concentration of antibiotics. The marrow was flushed out with cultured medium and expelled from a 5 ml syringe through a 23 gauge needle [16,17]. The marrow suspension was then transferred to tissue culture flasks containing 5 ml DMEM supplemented with 100 μg/ml penicillin–streptomycin (Gibco-BRL, Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 0.2 mM L-ascorbic acid phosphate magnesium salt *n*-hydrate (AscP; WAKO, Osaka, Japan), and 10 ng/ml basic fibroblast growth factor (b-FGF; Kaken, Tokyo, Japan). Nonadherent cells were removed from cultures after 4 days by a series of phosphate buffered saline (PBS) washes and subsequent medium changes. Adherent cells were expanded as monolayer cultures in a 5% CO_2/95% air atmosphere at 37 °C with medium changes every 3 days until confluent. The cells were then dissociated with trypsin and subcultured in 6-well culture dishes at a plating density of 5×10^5 cells/dish for additional 2–3 days.

Cloning of mouse sox9 cDNA. Total RNA was isolated from femora and tibiae of 18 day ICR mice using Trizol reagent (Gibco-BRL, Life Technologies, Grand Island, NY, USA). Cell constructs were transferred to a 1.5 ml microcentrifuge tube and were dissociated in 1 ml Trizol. After extraction with chloroform and precipitation with isopropanol, the total RNA was used as a template for reverse transcription into cDNA by Reverse Transcription System (Promega). cDNA was amplified by PCR with oligonucleotide primers (forward primer 5′-CTTCTCGCCTTTCCCGGCCA-3′, reverse primer 5′-GATCAGCTCTGTCACCATAGC-3′) complementary to regions of mouse full-length *sox9* cDNA. The PCR products were subcloned into the plasmid vector (pCR 2.1-TOPO vector; Invitrogen Life Technologies, USA) in accordance with manufacturer's instructions and double-stranded plasmid DNA was sequenced with BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) using the primers identical with those used for PCR amplification. The double-stranded plasmid DNA was cut with *Eco*RI and a full-length *sox9* cDNA was obtained.

Gene constructs and transfection. A 1.5-kb full-length *sox9* cDNA was inserted into pIRES2-EGFP vector (BD Biosciences Clontech, Palo Alto, CA, USA) in front of the EGFP coding region at the *Eco*RI site by using DNA Ligation Kit Ver.2 (Takara Biomedicals, Shiga, Japan). Inserted *sox9* sequences were confirmed by DNA sequencing. The gene constructs containing full-length *sox9* cDNA and EGFP coding region were transfected into subcultured MSCs in 6-well culture dishes of 80–90% confluency with LipofectAMINE 2000 reagent (Invitrogen Life Technologies, USA) by manufacturer's instructions. Expression of EGFP was observed and photographed under a fluorescence microscope (Olympus BX60; Olympus, Tokyo, Japan) using a blue filter (NIBA).

In vitro micromass culture. Tranfected cells were cultured for additional 2–3 days in culture medium containing Kanamycin for selection. The cells were grown to confluence, then trypsinized with trypsin–EDTA (Sigma, St. Louis, MO, USA) for 5 min at 37 °C, and resuspended at a concentration of 5×10^5 cells/ml in the defined medium. A 20 μl drop of this cell suspension was placed on a coverglass (32 mm diameter; Matsunami, Osaka, Japan) in a 6-well tissue culture dish, the cells were allowed to adhere to each other for 2 h at 37 °C, and then the defined medium was added gently back to the culture. Medium changes were carried out twice a week up to 14 days. Cells in micromass culture without gene transfection served as a control.

In vivo transplantation. Transfected cells were cultured for an additional 2–3 days in culture medium containing Kanamycin for selection. These cells were trypsinized, counted, and suspended in standard medium at the concentration of 5.0×10^5/100 μl, and then loaded into the diffusion chambers (0.45 μm pore size; Millipore, Bedford, MA, USA). Chamber embedded cells without gene transfection served as a control. Both the chambers with transfected cells and control cells were subcutaneously implanted into the dorsal side of athymic mice in pockets formed by blunt dissection. The mice were sacrificed at 4 and 8 weeks postimplantation and analyzed for chondrogenesis.

Histology and immnohistochemistry. Specimens were harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. Serial sections cut by 6 μm were stained with Alcian blue for proteoglycan detection and observed by bright field light microscopy. Staining of extracellular space is characteristic of the deposition of a cartilage matrix. For immunohistochemistry, serial sections were used for detection of type II collagens and type X collagens with specific antibody. The serial sections were deparaffinized, washed in phosphate-buffered saline (PBS), and then pretreated with 10% rabbit serum albumin for 10 min at room temperature to block nonspecific reactions. Type II collagen was immunolocalized with a goat polyclonal antibody C-19 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After a 1-h incubation in the primary antibody, sections were rinsed in PBS, and then incubated with peroxidase-labeled affinity purified antibody to goat IgG (1:100; KPL, Guildford, UK). Signal was developed as the brown reaction product of peroxidase substrate 3,3′-diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) and H_2O_2. Type X collagen was immunolocalized with a rat polyclonal antibody LB-0092 (1:100; S.L.S., Japan). For a negative control, the primary antibody was omitted.

Results

Reporter gene constructs containing mouse sox9 cDNA

pIRES2-EGFP vector ligated with the mouse full-length *sox9* cDNA was digested with *Eco*RI and

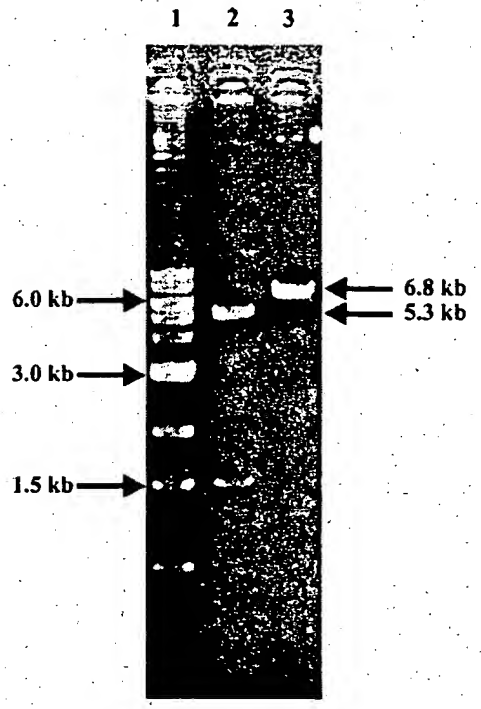

Fig. 1. Agarose gel electrophoresis after restriction enzyme digestion of pIRES2-EGFP vector containing the mouse full-length *sox9* cDNA. The 1.5-kb band was confirmed by *Eco*RI digestion. Lane 1: Marker; Lane 2: *Eco*RI digestion; Lane 3: *Sal*I digestion.

electrophoresed on the 1% agarose gel. A 1.5-kb band was observed (Fig. 1) and the sequences of this band were confirmed by DNA sequencing (data not shown).

Micromass culture: histologic and immunohistochemical findings

The micromass culture developed a multilayered matrix-rich morphology during culture and showed an increased proteoglycan-rich extracellular matrix histologically. Alcian blue staining, indicating the presence of highly negative charged sulfated proteoglycans, was observed at the central region of the micromass culture after 14 days in culture. The densely stained matrix was spatially concentrated in the micromass aggregates, and was considerably less in the surrounding regions where the fibroblast-like cells were in a confluent monolayer (Fig. 2A). In the MSCs without *sox9*-gene transfection, Alcian blue staining was not seen. Type II collagen was colocalized in the regions with the most intense Alcian blue staining, however, type X collagen staining was not detected. GFP expressing cells were observed comparatively at the central regions of the micromass culture where Alcian blue staining was positive (Fig. 2B).

In vivo transplantation: histologic and immunohistochemical findings

On the diffusion chamber loaded with *sox9* transfected cells, a whitish spherical tissue of about 2 mm in diameter was macroscopically observed after 4 weeks implantation (Fig. 3). More massive tissue and comparatively thick membraneous tissue were observed after 8 weeks implantation. Histological examinations demonstrated that a large portion of the inner area of the mass was more sparsely populated with round-shaped cells, and the densely stained matrix was present. Alcian blue as well as type II collagen were positively stained in the extracellular matrix of the mass but type X collagen was not detected (Figs. 4A–C). GFP expressing cells were also seen in the central regions of the newly formed tissue (Fig. 4D). On the other hand, only a thin membraneous tissue had adhered on the chambers loaded with non-transfected MSCs after 4 and 8 weeks implantation. Histologically, it was defined as a fat-like tissue that consisted of the accumulated cells with lipid-rich vacuoles.

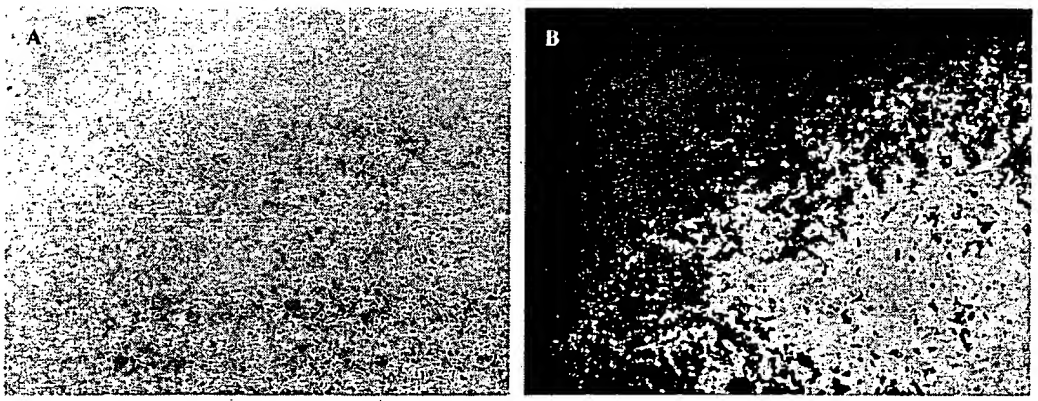

Fig. 2. High density micromass culture of *sox9* transfected MSCs indicating positive Alcian blue staining (A) and type II collagen immunostaining within the micromass aggregate. GFP expressing cells were also observed in the central region of the micromass (B).

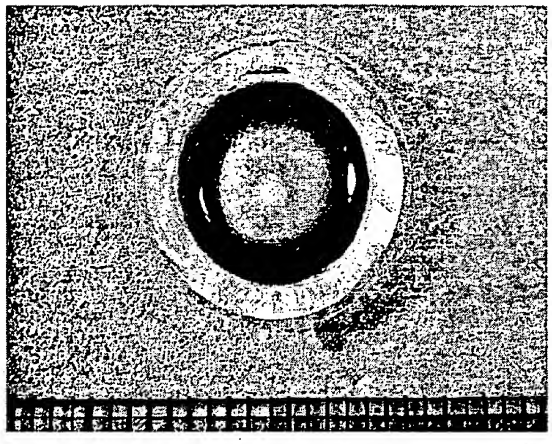

Fig. 3. Massive tissue formation on the diffusion chamber after 4 weeks transplantation of *sox9* transfected MSCs into subcutaneous resion of athymic mice.

Discussion

A wide variety of techniques and reagents are used to deliver nucleic acid into eukaryotic cells. These nucleic acids interact with cellular components and result in gene expression and protein synthesis. DNA delivery by virus infection is the most commonly used for experiments in its high transfection efficiencies, but biosafety problems would arise in clinical applications. On the other hand, DNA delivery by lipofection is less toxic and a simple method requiring only a few reagents including plasmid DNA containing the gene of interest under the control of a strong promoter. Recently, its simplicity and the numerous advances that have increased its level of expression efficiency have made lipofection a more popular method for nucleic acid delivery [18]. In this study, DNA delivery by lipofection instead of virus infection was applied for a potential use of tissue engineering in clinical feasibility. Although it has been recognized that the transfection efficiencies are relatively low in lipofection, numerous GFP expressing cells were observed within the micromass aggregates, indicating that reporter gene constructs can be successfully transfected to MSCs by this method. Transfection efficiencies of *sox9* gene by lipofection were satisfactory for chondrogenesis of MSCs.

Two fundamental approaches of delivering therapeutic genes to the muscloskeletal system currently exist: direct (in vivo) gene therapy and ex vivo gene therapy. The latter consists of tissue biopsy, cell isolation and

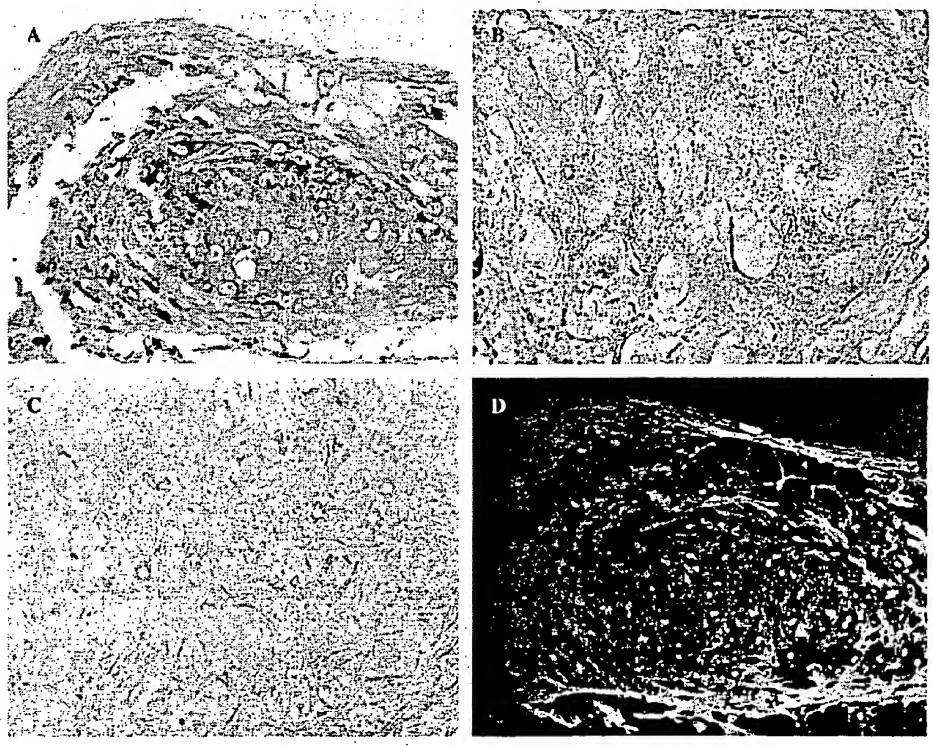

Fig. 4. Specimens from the massive tissue formed on the diffusion chamber demonstrating more sparsely populated round-shaped cells in the central region where both Alcian blue and type II collagen were positively stained (A, B) while type X collagen was not stained (C). GFP expressing cells were also observed in the specimens (D).

expansion in culture, in vitro genetic manipulation, and reintroduction of the genetically manipulated cells into the host [19]. The ex vivo approach could prevent risks relating to direct gene therapy because the manipulated cells may be tested in vitro for transduction efficiency and for tumorigenicity before introduction into the host. In this regard, ex vivo manipulation of *sox9* gene retains an advantage for future clinical applications of inducing cartilage formation.

Bone marrow is considered to be a suitable donor tissue in the cell-mediated gene therapy for cartilage regeneration since it can be obtained from a comparatively small amount of illiac crest biopsy and contains multipotent mesenchymal stem cells. MSCs have the ability to differentiate along multiple connective tissue lineages including bone, cartilage, tendon, ligament, muscle, and fat [5–10,20]. Several investigators have reported in vitro chondrogenic differentiation of MSCs in a pellet micromass culture with the defined medium containing bioactive factors such as dexamethasone and TGF-βs [6–8]. However, a short half-life of these bioactive factors and a loss of concentration in cultures have limited clinical applications. We have developed more simple and useful culture system that induces in vitro chondrogenesis of mouse MSCs by overexpression of *sox9* gene without addition of bioactive factors. Concentrated GFP expressing cells in vitro micromass culture and in vivo specimens demonstrated that longer effects of *sox9* protein could be expected in our method.

Several studies have shown that co-expression of long form *sox5* (L-*sox5*), *sox6* in addition to *sox9* is essential for chondrogenic differentiation of MSCs [21,22]. Although co-transfection of these genes into MSCs may be more effective in chondrogenesis, gene therapy transducing more than two genes is complicated and has less clinical feasibilities. In this study, overexpression of *sox9* gene in combination of micromass culture of MSCs induced chondrogenic differentiation in vitro and cartilage formation in vivo. The exact role of overexpressed *sox9* gene in the conversion of mesenchymal precursor cells into differentiated chondrocytes is still unknown, but a high density micromass culture may compensate for the necessity of co-factors, L-*sox5* and *sox6*.

In the growth plate of endochondral bones, three different layers of chondrocytes can be seen: resting chondrocytes, proliferating chondrocytes, and hypertrophic chondrocytes. Hypertrophic chondrocytes express high levels of alkaline phosphatase (ALP), type X collagen and diminished levels of type II collagen, and are predisposed to undergo apoptotic cell death for new bone formation (endochondral ossification). Parathyroid hormone-related peptide (PTHrP) is a negative regulator of chondrocyte maturation from prehypertrophic chondrocytes to hypertrophic chondrocytes [23]. Recently, several authors reported that *sox9* inhibits the maturation of prehypertrophic chondrocytes to hypertrophic chondrocytes by mediating the PTHrP signaling [24,25]. In this study both in vitro micromass aggregates and in vivo cartilage tissues, type II collagen was positively stained, whereas no immunostaining of type X collagen was observed. These results indicated that *sox9* helps maintain the chondrocyte phenotype to prevent promoting terminal differentiation. Therefore, *sox9* gene transfection to MSCs could be very useful for repairing the articular cartilage defect in keeping the function of the hyaline cartilage.

Fat-like tissue formation was observed on the chamber in transplantation of non-transfected MSCs. Since the chamber was transplanted in the subcutaneous fatty area of athymic mice, adpocyte inductive molecules factor such as PPARγ2 may have flowed into the chamber and induced adipogenesis of the multipotent mesenchymal stem cells [8]. On the other hand, successful development of cartilage tissue in the diffusion chamber was obtained by transplantation of *sox9* transfected MSCs, irrespective of an environment of transplanted sites. These *sox9* transfected cells independently differentiated into chondrocytes and secreted extracellular matrix proteins characteristic of cartilage since host cell components cannot flow into the chamber. More effective chondrogenesis may be expected by transplantation of *sox9*-transfected MSCs to the articular surface where hyaline cartilage was damaged. In conclusion, MSCs overexpressing *sox9* gene strongly supported the feasibility of a cell-mediated gene therapy approach for osteoarthritis, which is the most frequently encountered disease with cartilage damage among the orthopedic disease.

References

[1] S. Wakitani, T. Goto, R.G. Young, J.M. Mansour, V.M. Goldberg, A.I. Caplan, Repair of large full-thickness articular cartilage defects with allograft articular chondrocytes embedded in a collagen gel, Tissue Eng. 4 (1998) 429–444.

[2] M. Brittberg, A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson, L. Peterson, Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation, N. Engl. J. Med. 331 (1994) 889–895.

[3] P.D. Benya, J.D. Shaffer, De-differentiated chondrocytes re-express the differentiated collagen phenotype when cultured in agarose gel, Cell 30 (1982) 215–224.

[4] K. Elima, E. Vuorio, Expression of mRNAs for collagens and other matrix components in dedifferentiating and redifferentiating human chondrocytes in culture, FEBS Lett. 258 (1989) 195–198.

[5] A.I. Caplan, Mesenchymal stem cells, J. Orthop. Res. 9 (1991) 641–650.

[6] B. Johnstone, T.M. Hering, A.I. Caplan, V.B. Goldberg, J.U. Yoo, In vitro chondrogenesis of bone marrow-derived mesen- chymal progenitor cells, Exp. Cell. Res. 238 (1998) 265–272.

[7] A.M. Mackay, S.C. Beck, J.M. Murphy, F.P. Barry, C.O. Chichester, M.F. Pittenger, Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow, Tissue Eng. 4 (1998) 415–428.

[8] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 248 (1999) 143–147.
[9] T. Saito, J.E. Dennis, D.P. Lennon, R.G. Young, A.I. Caplan, Myogenic expression of mesenchymal stem cells within myotubes of mdx mice in vitro and in vivo, Tissue Eng. 1 (1995) 327–343.
[10] R.G. Young, D.L. Butler, W. Weber, A.I. Caplan, Use of mesenchymal stem cells in a collagen matrix for achilles tendon repair, J. Orthop. Res. 16 (1998) 406–413.
[11] E. Wright, M.R. Hargrave, J. Christiansen, L. Cooper, J. Kun, T. Evans, U. Gangadharan, A. Greenfield, P. Koopman, The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos, Nat. Genet. 9 (1995) 15–20.
[12] D.M. Bell, K.K.H. Leung, S.C. Wheatley, L.J. Ng, S. Zhou, W.L. Ling, M.H. Sham, P. Koopman, P.P.L. Tam, K.S.E. Cheah, Sox9 directly regulates the type-II collagen gene, Nat. Genet. 16 (1997) 174–178.
[13] V. Lefebvre, W. Huang, V.R. Harley, P.N. Goodfellow, B. de Crombrugghe, Sox9 is a potent activator of the chondrocyte-specific enhancer of the proα1(II) collagen gene, Mol. Cell. Biol. 17 (1997) 2336–2346.
[14] I. Sekiya, K. Tsuji, P. Koopman, H. Watanabe, Y. Yamada, K. Shinomiya, A. Nifuji, M. Noda, Sox9 enhances aggrecan gene promotor/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell, TC6, J. Biol. Chem. 275 (2000) 10738–10744.
[15] W. Bi, J.M. Deng, Z. Zhang, R.R. Behringer, B. de Crombrugghe, Sox9 is required for cartilage formation, Nat. Genet. 22 (1999) 85–89.
[16] C. Maniatopoulos, J. Sodek, A.H. Melcher, Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats, Cell Tissue Res. 254 (1998) 317–330.
[17] Y. Takamine, H. Tsuchiya, T. Kitakoji, K. Kurita, Y. Ono, Y. Ohshima, H. Kitoh, N. Ishiguro, H. Iwata, Distraction osteogenesis enhanced by osteoblast-like cells and collagen gel, Clin. Orthop. 399 (2002) 240–246.
[18] B. Micka, B. Trojaneck, S. Niemitz, P. Lefterova, S. Kruopis, D. Huhn, B. Wittig, D. Schadendorf, I.G.H. Schmidt-Wolf, Comparison of non-viral transfection methods in melanoma cell primary cultures, Cytokine 12 (2000) 828–833.
[19] D.S. Musgrave, P. Bosch, J.Y. Lee, D. Pelinkovic, S.C. Ghivizzani, J. Whalen, C. Niyibizi, J. Huard, Ex vivo gene therapy to produce bone using different cell types, Clin. Orthop. 378 (2000) 290–305.
[20] P. Bianco, P.G. Robey, Stem cells in tissue engineering, Nature 414 (2001) 118–121.
[21] V. Lefebvre, P. Li, B. de Crombrugghe, A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene, EMBO J. 17 (1998) 18–33.
[22] V. Lefebvre, R.R. Behringer, B. de Crombrugghe, L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway, Osteoarthritis Cartilage 9 (2001) S69–S75.
[23] U. Chung, E. Schipani, A.P. McMahon, H.M. Kronenberg, Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development, J. Clin. Invest. 107 (2001) 295–304.
[24] B. de Crombrugghe, V. Lefebvre, K. Nakashima, Regulatory mechanisms in the pathways of cartilage and bone formation, Curr. Opin. Cell. Biol. 13 (2001) 721–727.
[25] W. Huang, U. Chung, H.M. Kronenberg, B. de Crombrugghe, The chondrogenic transcription factor Sox9 is a target of signaling by the parathyroid hormone-related peptide in the growth plate of endochondral bones, Proc. Natl. Acad. Sci. USA 98 (2001) 160–165.